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Fluorescent-Serological Diagnosis of Bovine Leukosis

FLUORESCENT-SEROLOGICAL DIAGNOSIS OF BOVINE LEUKOSIS

[Following is the translation of an article by A. Tolle of the Veterinary Institute of the University of Goettingen, in the German-language periodical Deutsche tieraerztliche Wochenschrift (German Veterinary Weekly), Vol 68, No 7, 1 April, 1961, pages 193-202.]

I. INTRODUCTION

Lately the suspicion that bovine leukosis is caused by a transferable agent becomes more and more likely. Several authors (Lobberstein et al. (40,41), Fr. and H. Schottler (134), Knuth (76), Gotze, Rosenberg and Ziegenhagen (49)) were able to cause characteristic hematological changes of leukosis by transfer of milk, blood, tissue pulp or feces from leukotic cattle to cattle of the same species. Three of the seven animals infected by Gotze et al. even became sick with humorous leukosis. No transfer with cell-free material has succeeded so far. The systematic use of the "leukosis key" developed by Gotze, Rosenberger and Ziegenhagen (46,49) has enabled the authors to make the following observations concerning the spread of bovine leukosis, which leave hardly any other explanation but communicability:

1. Bovine leukosis spreads systematically from the East to the West of Germany. Similar observations have been made in Denmark (Bendixen [7] and in Sweden (Hjarre [64]).

2. Leukosis is introduced in herds by the purchase of animals from the East, or by roaming animals, and it manifests itself then for years or decades in these herds.

3. The further spread of leukosis from herd to herd is similar to the spread of chronic infection diseases. It is most frequently spread through the purchase of breeding animals.

4. When cattle from leukosis-free regions are introduced in herds where there is leukosis, sooner or later a number of these healthy animals become sick.

5. Leukosis occurs on average or large size farms under varying circumstances of nutrition, treatment and management.

6. The infection theory is also supported by the fact that leukosis disappears completely after strongly diseased herds have been eliminated and replaced with healthy animals, after the barn had been well cleaned and disinfected.

Indeed Bjarre 1958 (64) states that tumor material from cattle shows in chromatographic fractionation the same changes as in fowl. The electronmicroscope showed characteristic particles in the "virus fraction". Montemagno Papoarella and Catallani (1957)(108) vaccinated chicken embryos with filtered lymph gland material of a calf suffering from lymphatic leukosis, and in 20 more passages made an agent that they claim to be the exciter of bovine lymphadenosis. Montemagno (1958) (106) transferred allantoic fluid from infected chicken embryos to two healthy calves. After three weeks there was a feverish, general illness which did not react to antibiotics, and after two months there was a temporary swelling of the lymph glands and hematologically-positive values. In a later publication, Montemagno (1958) (106) describes the leucocytogenic effect of the plasma of leukotic cattle after injection in laboratory animals. In 1959, Papparella (122) published a morphological description of the virus and three electron microscope photographs.

The slow spread of bovine leukosis — which is hidden because of the chronic course of its infectious character — can be taken as a sign that the virus has little resistance outside the host animal. Gotze, Rosenberger and Ziegenhagen (50) consider the following possible methods of transfer:

1. Diaplacentaltransfer

2. Transfer through milk.

3. Transfer by contact with milk, blood, amniotic fluid or feces of animals with hematologic or tumorous leukosis.

The first way of infection can, according to the authors, be considered proven while the other possibilities still lack sure experimental proof.

Although the pathological-anatomical and histological changes in the case of bovine leukosis have been investigated and described thoroughly by Knuth and du Toit (77), Knuth and Volkmann (75), Dobberstein et.al. (38,39,40,41) and others, we still know little about the pathogenetic questions. How does the infection occur? Is the virus alone responsible for causing the disease, or are other conditions necessary? How does the virus behave in the body of the animal? How is it excreted? How well

does it survive in the outside world, when attacked by drugs and disinfectants? Can the virus be transferred to other species of animals? All these questions can be answered only when we succeed in following the agent on its way. A sure knowledge of the pathogenesis and an early diagnosis are therefore essential for a possible nationwide attack against this epidemic which is constantly spreading in Northern Germany.

The hematological control of the herds enables us without doubt to have a good enzotologic survey, but basically this method gives only the pathological-histological sequence, and not the cause of the illness, when we consider blood to be tissue with fluid with liquid intracellular substance. Besides, we can of course recognize only leukemic stages of disease, and it is very difficult to recognize lymphocytic hyperplasia from non-leukotic causes. Everybody who is familiar with hematological control knows the considerable spread in the results of repeated investigation. Finally, the use of hematological methods in general mass investigations takes a tremendous amount of work for which the personnel is, under present circumstances, hardly available. In spite of these known arguments the "leukosis key" of Getze, Rosenberger and Ziegenhagen (46) has stood the test of practical application, and it has given valuable impulses to the leukosis research. Especially, it enabled us to recognize the fact that the disease goes through two phases: the hematological first stage and the tumorous clinical stage. The "leukosis key" is so far certainly the best available method of diagnosis. In our own investigations which we will next describe we compare the results obtained with the leukosis key with those from the fluorescent-serological method. The principle of using fluorescing antibodies developed by Coons and co-workers (25, 26, 31, 33), seemed, as a virological, immunological method suitable for recognizing the leukosis agent. We next describe the first experiences with this method.

IV. THE IDENTIFICATION OF ANTIGENS WITH FLUORESCING ANTIBODIES

The binding of antigens to their corresponding antibodies is indirectly shown by the classical method of serology. The microscopic appearance of agglutination, precipitation and complement binding reaction is a physical and chemical result of the binding, just like the toxin and virus-neutralisation are the biological result of the binding.

The method of marking antibodies with fluorescing dyes, developed by Coons and co-workers, makes it possible to see the antigen-antibody binding optically. It combines the specificity of serological reactions with the resolution of the microscope. Since this method has not yet been described in German veterinarian publications, we will give a short survey of the principle, the results obtained so far by other authors, and its technical application.

The principle of Coons method is that the albumen molecules of a

purified antibody solution in vitro can make a chemical reaction with one or two molecules of a fluorescing dye. In this way, the fluorescing dye is conjugated to the eggwhite, without influence on its serological specificity. When the marked antibodies are allowed to react with a tissue cross section or a smear or streak sample containing corresponding antigens, then the fluorescing antibodies are fixed by the corresponding antigens. All antibodies which are not bound can wash off. Wherever the antigen-antibody binding has occurred, we can see the resulting fluorescence under the fluorescence microscope.

This method is a significant contribution to immunological research and diagnosis. It has made possible the localisation of the places where antibodies are formed (2, 29, 30, 32, 121, 82, 153, 154, 157, 158). The protein transfer from mother to fetus was proved (5, 92). The presence of injected, foreign antigens has been studied (28, 45, 91, 147). The bodies' own antigens and antibodies were analyzed (22, 23, 35, 62, 72, 73, 95, 96, 97, 98, 99, 94, 100, 120, 135). In immune-histochemical research, hormones and enzymes were shown (4, 11, 61, 63, 80, 101); blood group substances were also demonstrated (1, 67, 68, 138). For medical microbiology, the possibility of differentiation of infectious agents is extremely interesting. The following microorganisms have so far been investigated with the fluorescent serological method: Amebas (55,66), Ciliates (6), Anaplasma (120) Toxoplasma (18, 19, 52, 53, 54), Fungi (42, 57, 78, 146), Pneumococcus (26, 70), Streptococcus (58, 59, 71, 104, 105, 132, 133), Coli bacteria (51, 123, 143, 152), Salmonella (34, 141, 142), Shigella (69, 79), Proteus bacteria (125), Pasteurella (155, 156), Haemophilia bacteria (126), Malleomyces pseudomally (102, 103, 140), Bac.anthracis (83), Pansen bacteria (65), Treponema (36, 137), Leptospires (110, 136), Rickettsia (130), further the virus of human influenza (14, 60, 86, 150), Parotitis epidemica (21, 27, 148, 149), measles (3), human skin papilloma (13), Shope's rabbit papilloma (118), fret influenza (84), Hepatitis contagiosa canis (24), Psittacosis (17), classical (15) and atypical bird plague (43), Herpes simplex (12, 119, 131), human atypical pneumonia (85, 87, 89), Egypt 101 (116), rabies (56), Poliomyelitis (16, 31, 139), dogs distemper (88, 109), Vaccinia (117), hoof-and-mouth disease (112), Teschener swine paralyses (111), and the Herpes Zoster (151).

It is remarkable to observe that now fluorescence immunological kinds of virus can be identified immediately without considerable technical laboratory equipment. According to the principle of the method, they can not only be represented microscopically, but they can also be serologically analysed according to their specific antigen nature. This gives us a practical method for the solution problems of the pathogenesis of virus infections. We can also diagnose those virus diseases which have no real cytopathogenic effects. The localisation of the virus in the organism can be determined immediately.

An excellent survey of the application of fluorescing antibodies

for immune histochemical research has been published by Coons (31, 33). Liu (90) published in 1960 a survey of the use of the method with virus - and rickettsia infections. In Germany, the method of fluorescent serology has been pointed out by Poetschke /see note/ and co-workers (124).

/Note/ Here, we wish to thank Prof. Dr. Gerd Poetschke, Munchen, for valuable advice during preliminary technical difficulties.

Dye: Originally Coons and co-workers used the dye fluorescein isocyanate. In that case the coupling with the eggwhite body occurs via the C-N-O group:

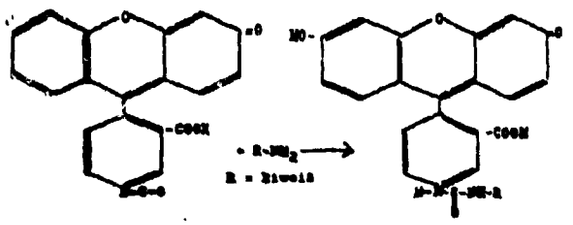

Fig. 1. The coupling of fluorescein isocyanate to albumin.

The synthesis of the fluorescein isocyanate is however difficult and the compound is also very unstable. Coons has it made, as needed and couples it immediately to the antibodies. The introduction of the stable and easy-to-store fluorescein isothiocyanate by Riggs et. al. (128) was therefore a welcome improvement. The authors have described the synthesis, but the dye can be obtained commercially. With fluorescein isothiocyanate the coupling occurs over the S-C-N-group:

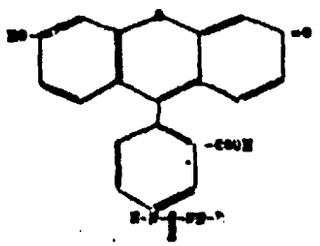

Fig. 2. Fluorescein isothiocyanate coupled to albumin. (R = albumin).

There have been many attempts to make other dyes for the coupling to proteins. The most frequently used ones are sulfonic acids, which are chlorated and coupled to the albumin via a sulfonamide compound. Clayton (23) used 1-dimethyl -amino-5-naphthaline sulfonic acid. Chadwick et al (20) and Uehleke (144) marked proteins with sulforhodamin-B. Uehleke also recommends 3-hydroxypyren-5.8.10-trisulfonic

acid as highly suitable for protein conjugation. Mayersback (93) introduced the acid chloride of 1-dimethylnaphthyl-aminosulfonic acid -5. The conjugation of this dye to a protein goes via a sulfonamide compound:

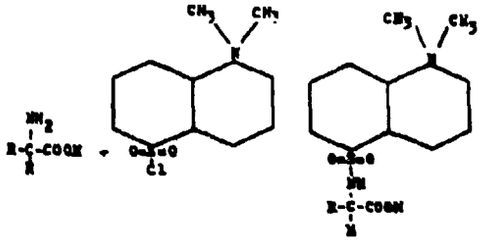

Fig. 3. Coupling of the acid chloride of 1-dimethylnaphthylamin-sulfonic acid-5 to albumin according to Mayersbach (93). R = albumin.

The sulfochlorides give stable compounds with albumin. Finally, Chadwich, McEntegart and Nairn (20) coupled "lissamine Flavine FFS" to antibodies over a diazo compound.

The use of dyes with emission spectra of different wavelength makes it possible to localise several antigens in one sample. Clayton (23) was the first one to do this.

The fluorescein derivates and the hydroxy-pyren-sulfonic acids have a sharp, green fluorescence; dimethylaminonaphthaline sulfo acid dimethylnaphthylaminosulfo acid give a sharp yellow fluorescence and sulforhodamin-B lights orange-red. Compared with the other dyes, sulforhodamin-B is well suited for contrast coloring.

For our research we used fluoresceinisothiocyanate.

III. OUR OWN RESEARCH

A. Methods

1. The adaptation of leukosis agent under experimental control for serological identification, we tried first to adapt bovine leukosis to baby mice. Mice are easily-managed experimental animals with a short life span, rapid sequence of generations and high reproduction rate. When the parent animals have not been in contact with tumor antigen, so that they cannot transfer antibodies to embryos or young animals, the baby mice are good nutrients for the tumor antigen, since they have no defense. The tumor antigen can be of heterogenous origin.

Mice of the American pure breed N.M.R.I. (Naval Medical Research Institute, Bethesda, U.S.A.) were used. According to information from the leaders of the supplying central institute for the growing of experimental animals, spontaneous leukosis did not occur in this species. In order to eliminate the influence of a possible antibody formation and its diaplacental and colostral transfer to embryos and young animals, only one litter of each couple of parents was used.

Baby mice 12 to 48 hours old were given a subcutaneous, intramuscular injection of fresh leukosis material, at body temperature, from a young recently-killed cow. The material had been homogenized in Griffith tubes.

Two to three weeks later, most baby mice were killed, but a few were kept for further observation. Liver, milt and lymphatic tissue of the killed mice were then homogenized in Griffith tubes, and after diluting with some phosphate buffer according to Klone, (74) the cell-containing material was reinjected in newly-born mice.

2. The conjugation of the dye to purified globulin.

Serum gamma globulin was purified by precipitation with ammonium sulfate and subsequent dialyses (Dickel (32)).

The albumin content of the so purified globulin solution was determined with the micro kjeldahl method. For weak serums, we recommend concentration by liophilization or dialyses.

Coupling: Per mg. of protein, we needed 0.05 mg. fluorescein isothiocyanate. The dye was dissolved in dried acetone, in the proportion of 10 mg. dye to 1 cc. acetone. These substances were mixed in an Erlenmeyer flask, cooled to 0°C and stirred mechanically:

10 cc. 0.87% NaCl solution (0.15 M).

3 cc. carbonate-bicarbonate buffer (0.5 M) pH = 9.0.

2 cc acetone (dried over $CaSO_4$).

To this cool mixture, we added, while stirring, 10 cc of the cooled globulin fraction with known protein content. Subsequently, we added drop by drop (0.1 cc in about 15 min.) 0.05 mg. of the dye dissolved in acetone per mg. of protein. After addition of the dye, the solution may not be acid. All the same, some albumin always precipitates. The solution containing dye must be stirred for 18 hours at 0-2°C and is subsequently dialysed at the same temperature against 0.15 M NaCl solution (pH = 7.4), until the repeatedly-changed dialysation fluid shows no fluorescence in UV light. The coupled dye is put in reaction tubes and

stored in deep freeze (-36°C) until used.

Elimination of non specific coloring

The removal of dye that has not sufficiently been removed by dialysis is of great importance for the use of fluorescent antibody solutions. The coupled globulin which was stored in the deep freeze was therefore freed of precipitated albumin by centrifuge and then absorbed on surface-active substances. Specially suitable for this are acetone-dried pulverised mouse or rabbit liver or bone marrow or active carbon, which do not dissolve in NaCl solutions. About 50 mg. pulver is added per cc. conjugate, it is incubated and shaken for 1 hour and then centrifuged at 15,000 r.p.m. at low temperature.

3. The dye conjugation to purified anti-leukosis globulin for the direct identification of antigen.

According to the above-mentioned method the purified gamma-globulin from the serum of cattle ill with leukosis is coupled with fluorescein isothiocyanate. This method, however, was found to be unsuitable for our investigations because apparently the antibodies against leukosis agents in the blood serum have a very weak concentration, and they are still further reduced by the coupling procedure.

In order to avoid this weakening of the leukosis antibodies, the indirect identification of antigens with the coupled anti-globulin was tried.

4. The dye conjugation to purified anti-globulin for the indirect identification of antigen.

This method makes it possible to make an antigen-antibody coupling visible with a dye-coupled anti-globulin (Weller and Coons (151)). In the layering antigen-antibody-anti-antibody, the antibody which is not marked works as a bridge and is at the same time on one side an antibody and on the other side an antigen (see schematic representation).

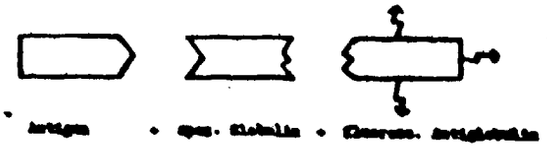

Fig. 4. Schematic representation of the anti-globulin method for the indirect identification of antigen.

This method is specially very suitable for working with weak specific sera.

In our own research we obtained a high grade of anti-globulin by intravenously injecting carefully purified bovine globulin in rabbits. The intravenous injections are supported by intramuscular injections of globulin and Freund's Adjuvans (74). The anti-globulin was purified as described above and coupled to fluorescein isothiocyanate.

5. Research with known antigen and unknown antibodies

As antigen we used smear samples of mouse tumors, as antibodies we used sera of cattle to be examined. Antigen-antibody bindings were proven by fluorescent rabbit-anti-cattle-gamma-globulin.

The main- and control samples were made according to the system of Table 1.

6. Research with unknown antigens and known antibodies

With this method we tried to identify the antigen in blood samples of cattle. As antibody we used the serum of a cow which had had leukosis for a long time, and which was under regular hematological control (H.B. Nr. 82331). The serum is permanently preserved by lyophilisation. The main sample and the control samples were made according to the system of Table 2.

Table 1

Schematic representation of the preparation of samples for the investigation of specific leukosis antibodies.

main experiment	1st. control	2nd. control	3rd control
1. object glass smear sample of leukotic mouse tumors			smear sample of healthy mouse milt.
2. drying in air, fixing for 15 min. with cold (-79°C) absolute alcohol			
3. treatment of 30 min. in humid room at +37°C with: patient's serum	positive leukosis serum	negative leukosis serum	patient's serum
4. 30 min. rinsing in buffered NaCl solution (0.15 M NaCl solution, containing 0.01 M phosphate, pH = 7.2).			
5. 30 min. in humid room at +37°C treatment with fluorescing rabbit-anti-cattle-gamma-globulin.			
6. 30 min. rinsing in buffered NaCl solution (see no. 4).			
result under UV microscope + or - fluorescence	required: +fluorescence	required: -fluorescence	required: -fluorescence

Table 2

Schematic representation of the preparation of samples for the investigation of leukotic specific antigen.

	main experiment	1st control	2nd control	3rd control
1.	citrate blood smear on object glass of: patient's	a leukosis positive cow	a leukosis negative cow	a leukosis positive cow
2.	drying in air, fixing for 15 min. in cold (-79°C) absolute alcohol.			
3.	treatment for 30 min. in humid room at +37°C with: anti-leukosis serum (H.Ko.Kr.82331)			serum of a healthy cow
4.	30 min. rinsing in a buffered NaCl solution (0.15 M NaCl solution, containing 0.01 M phosphate, pH = 7.2)			
5.	30 min. treatment in a humid room at +37°C with fluorescing rabbit-anti-cow-gamma-globulin.			
6.	30 min. rinsing in a buffered NaCl solution (as under 4).			
Result under UV microsc.	+ or - fluorescence	Required: +fluorescence	Required: -fluorescence	Required: -fluorescence

The smear samples are made by centrifuging ordinary citrate blood samples for 10 min. at 2500 r.p.m. after which the citrate plasma on top is mostly sucked off, so that only a thin layer of plasma remains above the layer of leukocytes of the cell sediment. With a curved platinum tool, a drop of the mixture of plasma-leukocytes-erythrocites is taken out and put on the object glass.

7. Technique of the fluorescence microscope.

Microscope: Large fluorescence attachment to the Zeiss photomicroscope, with high pressure mercury light source HB 200 (Osram).
Filter: UG 5,UG 2,2 x BG 12.
Eyepiece blocking filter: Orange blocking filter to be screwed into the eyepiece.
Aperture of the condenser: 1.3
Objective: Plan-Apochromat 100/1.32 oil.
Film: Agfa-Isopan-Rekord or Ilford.

8. Hematological investigation.

The total number of leukocytes was determined after treatment with

the methyl violet acetic acid in the counting chamber of Thoma. The differentiation of the leukocytes was done in a blood smear colored with the method of Pappenheim.

2. Material.

Full and citrate blood samples and smears for the hematological control were taken from 510 mixed-black lowland cows from 29 herds in Southern Lower Saxony, in which there was at least one case of tumorous leukosis. Most blood samples were taken and most hematological investigations done under the direction of Dr. Prange from the Organization for Epidemic Animal Diseases in Niedersachsen.
(Note: Here, we wish to thank the Director Dr. Seppin, Hannover, for putting the animal anti-epidemic service at our disposal for our research. For the tedious work of counting blood samples, I thank Dr. Prange. He is co-author of a forthcoming publication about the spread of bovine leukosis in Southern Lower Saxony.)

3. Results of the Investigations

1. The adaptation of the leukosis agents to baby mice.

With the previously explained technique, 497 mice were infected in three stages. After the third stage, there was a beginning tumor on the place of the injection of a 22 day old mouse. This tumor has been grown in many more stages, at the moment it is in its 15th stage. In the fourth stage, the tumor yield was only 3%. It increased to about 90% in the 15th stage. At the same time, the rate of growth of the vaccination tumors increased with the number of stages. Following the 9th stage, the tumor is clearly visible after 7 days. The following pictures show a leukosis vaccination tumor in a 16 day-old mouse. In the living mouse (Fig. 5.), we clearly see the strong tumorous swelling of the back side of the neck and the back (place of injection). In the dead mouse (Fig. 6), the nut-sized tumor with its sharp limits on the back side of the vertebra is shown as an open specimen. Histological investigation identified the very squashy tumor as lymphocytic hyperplasia.

An intended attempt to implant pieces of tumor from the sixth stage in grown mice had a negative result. After ten days the implanted part was completely resorbed. Implanted tumors from the ninth stage, however, have developed to growing tumors in grown mice, when just before the implantation, 1 mg. of cortisone was injected subcutaneously. Cortisone apparently prevented a defense reaction. A grown mouse which was treated in this way is pictured in Fig. 7.

Fig. 5. Experimentally grown lymphocytic tumor in a 16-day old mouse.

Fig. 6. Open specimen of a nut-size tumor on the back side of the neck-chest-spinal column.

Fig. 7. Grown mouse with tumor growth after preliminary treatment with cortisone.

The first occurrence of a tumor in the place of injection of a young mouse and the gradual adaptation to baby mice together with the fluorescent serological identification with bovine leukosis serum of antigens in these tumors, make it likely that these transplantable

tumors are heterogeneous material from cows. But we can not completely exclude the possibility that mouse leukosis started spontaneously, or was triggered by the injection. At this moment, there is no method to disprove this possibility.

2. The fluorescent-serological identification of a leukosis antigen.

In smear samples of mouse tumors and blood samples of tumorous or hematologically leukotic cows and in cows which did not yet show any hematological changes, we found — with the previously mentioned technique — fluorescing point sources. On the basis of the controls, these mostly represent the marking of a leukosis antigen (virus). The size of these marked particles ranges from the limit of the microscopic power to something similar to coccus (presumably virus agregates). They are present in blood plasma, in tumor intracellar fluid and also in and on lymphocites and erythrocites. Their brilliant, sharp, yellow fluorescence makes them prominently visible under microscopic observation. However it is often somewhat difficult to present in micro-photos, because the fluorescence decays considerably after long exposure, while the light strength of the smallest marked particles is not enough to darken the negative. We reproduce here 7 micro-photos. The size of the particles can be compared with the erythrocytes, which appear in a weak non-specific circle of light.

In addition to the controls mentioned in Table 1 and 2 — necessary for assurance of specificity — we investigated blood samples of sheep, goats, dogs, rabbits and guinea pigs. In none of the described blood samples did we see the described particles.

Fig. 8-10. Fluorescent-seriologically marked leukosis-specific particles (virus) in blood plasma of cows.

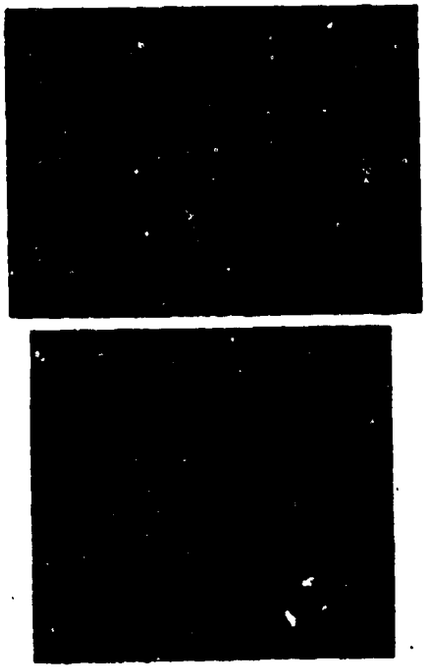

Fig. 11 and 12. The leukosis-specific particles (virus) are present both in plasma and on and in erythrocytes.

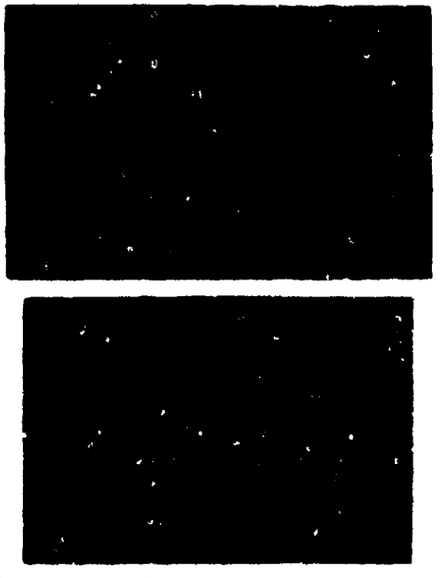

Fig. 13 and 14. Situation of the leukosis-specific particles only in the erythrocytes. In the cells the inclination to make(virus)aggregates is clearly visible.

3. Statistical evaluation of the method of investigation.

All the material can be arranged in two groups. In the first group (142 cows) the serum of the animals to be investigated was checked for leukosis specific antibodies with known tumor antigen. In the second group (368 cows) blood samples of the animals to be investigated were checked for leukosis specific antigen (virus) with known antibodies. For the comparison of the fluorescent-serological results with the results of the hematological control according to the leukosis key every group is subdivided into "animals more than 3 years old" and "animals less than 3 years old".

a). The correlation of the fluorescent-serological identification of leukosis-specific antibodies with the absolute number of lymphocyter.

/Note: The total number of leukocytes has also been evaluated for each group, but they are not published because of lack of space. They are proportional to the absolute number of lymphocytes. Those interested can request the tables from the author./

Table 3.

The correlation of absolute lymphocytic values with antibody identification on 118 cows older than 3 years.

Total number of lymphocytes	No. of animals	Fluorescent-serological positive abs.	%	questionable abs.	%	negative abs.	%
1,000 - 2,000	2	-	-	-	-	2	100.0
2,000 - 4,000	42	3	7.1	-	-	39	92.9
4,000 - 6,000	35	4	11.4	-	-	31	88.6
6,000 - 8,000	11	2	18.2	-	-	9	81.8
8,000 -10,000	7	3	42.9	-	-	4	57.1
10,000-12,000	5	2	40.0	-	-	3	60.0
12,000-14,000	7	5	71.4	-	-	2	28.6
14,000-16,000	4	3	75.0	-	-	1	25.0
16,000-18,000	1	-	-	-	-	1	100.0
18,000-20,000	2	1	50.0	-	-	1	50.0
beyond 20,000	2	2	100.0	-	-	-	-
	118	25				93	
Summary:							
up to 6,000	79	7	8.9	-	-	72	91.1
6,000-13,500	30	12	40.0	-	-	18	60.0
beyond 13,500	9	6	66.7	-	-	3	33.3

In this last table, the animals over three years old are subdivided

according to their number of lymphocytes and correlated with the result of the fluorescent-serological antibody investigation.

The 25 cows with positive fluorescent-serological reactions have an average of 11,409 lymphocytes; the 93 cows with negative reactions have 5,140 lymphocytes. The average difference is 6,269 lymphocytes. The t-test for the comparison of two average values could not be used for the statistical confirmation of this difference, because there is no normal distribution, but the variations are significantly different. Therefore we used the Wilcoxen-test: the formula is:

$$u = \frac{K - \frac{N_1 (N_1 + N_2 + 1)}{2}}{\sqrt{\frac{N_1 N_2 (N_1 + N_2 + 1)}{12}}}$$

N_1 = number of fluorescent-serologically positive cows

N_2 = number of fluorescent-serologically negative cows

K = sum of the negative sequence numbers.

This non-parametric test showed that the difference in the distribution of the fluorescent-serologically positive and negative animals against the lymphocytic values ($u = 21.7 \Rightarrow 3.37$) with $P < 0.01$ is highly significant.

The following table gives the correlation of the absolute lymphocytic values to the antibody identification in 24 cows younger than 3 years.

Table 4: The comparison of the absolute lymphocytic value with the antibody identification of 24 cows younger than 3 years.

total number of lymphocytes	number of animals	fluorescent-serological					
		positive		questionable		negative	
		abs.	%	abs.	%	abs.	%
2,000 - 4,000	1	-	-	-	-	1	100.0
4,000 - 6,000	12	-	-	-	-	12	100.0
6,000 - 8,000	9	-	-	-	-	9	100.0
8,000 -10,000	2	1	50.0	-	-	1	50.0
	24	1				23	

When we see this table we note that it is interesting that of the 24 young animals, only one had identifiable antibodies. The Wilcoxen test is here unnecessary.

b). The correlation of the antibody identification according

to the hematological identification with the leukosis key.

For this comparison, we omit the hematologically questionable cases. In the four-column table, we then get the following values:

		Fluorescent-serological results		
		+	-	
Hematological result according to the leukosis key	+	9	4	13
	-	12	101	113
		21	105	126

Result of the X^2-Test:

$X^2_{emp} = 28.4$

$X^2_{tab} = 10.82$ at 0.1% I.W. for 1 FG.

Corelation factor: $r = 0.476$

The correlation is therefore highly significant.

c). The correlation of the fluorescent-serological identification of leukosis-specific antigen (virus) to the absolute number of lymphocytes.

Table 5 contains the grouped lymphocytic values and the fluorescent-serological antigen identification for 312 cows older than 3 years.

Table 5. The correlation of the absolute lymphocytic values to the identification of leukosis-specific antigen in 312 cows older than 3 years.

Total number of lymphocytes	Number of animals	Fluorescent-serological positive abs.	positive %	questionable abs.	questionable %	negative abs.	negative %
1,000 - 2,000	13	9	69.2	1	7.7	3	23.1
2,000 - 4,000	82	37	45.1	7	8.6	38	46.3
4,000 - 6,000	71	25	35.2	8	11.3	38	53.5
6,000 - 8,000	31	15	48.4	6	19.4	10	32.2
8,000 -10,000	16	11	68.8	4	25.0	1	6.2
10,000-12,000	18	11	61.1	2	11.1	5	27.8
12,000-14,000	14	8	57.1	2	14.3	4	28.6
14,000-16,000	16	12	75.0	3	18.8	1	6.2
16,000-18,000	12	8	66.6	3	25.0	1	8.4
18,000-20,000	5	5	100.0	-	-	-	-
20,000-22,000	4	3	75.0	-	-	1	25.0
22,000-24,000	8	8	100.0	-	-	-	-
24,000-26,000	7	4	57.1	3	42.9	-	-
26,000-28,000	2	1	50.0	1	50.0	-	-
28,000-30,000	4	3	75.0	-	-	1	25.0
beyond 30,000	9	8	88.9	1	11.1	-	-
	312	168		41		103	
Summary:							
up to 6,000	166	71	42.8	16	9.6	79	47.6
6,000-13,500	74	41	55.4	14	18.9	19	25.7
beyond 13,500	72	56	77.8	11	15.3	5	6.9

The average number of lymphocytes in the positive cases is 11,564; of the negative cases 5,562. The difference is 6,002 lymphocytes.

According to the Wilcoxen test, the different distribution of the positive and negative cases compared with the number of lymphocytes is highly significant ($u = 19.0 = > 3.29$) with $= < 0.001$.

The young animals of this second research group are considered in Table 6.

Table 6. The connection of the absolute number of lymphocytes to the identification of leukosis-specific antigen in 56 young animals under 3 years.

total number of lymphocytes	number of animals	fluorescent-serological positive		questionable		negative	
		abs.	%	abs.	%	abs.	%
2,000 - 4,000	7	2	28.6	1	14.3	4	57.1
4,000 - 6,000	22	7	31.8	6	27.3	9	40.9
6,000 - 8,000	12	4	33.4	1	8.3	7	58.3
8,000 -10,000	7	4	57.1	2	28.6	1	14.3
10,000-12,000	3	3	100.0	-	-	-	-
12,000-14,000	3	1	33.4	-	-	2	66.6
14,000-16,000	-	-	-	-	-	-	-
16,000-18,000	2	2	100.0	-	-	-	-
	56	23		10		23	

Average number of lymphocytes in the fluorescent-serological cases: 9,062, in the negative cases: 6,190. The difference is 1872.

In the Wilcoxen test, we have $u = 1.38 = < 2.0$ and therefore not significant. The lack of significance in this group can be seen as proof that the hematological judgment of young animals is extraordinarily difficult.

d) The correlation of the antigen identification to the hematological judgment according to the leukosis key.

Just as under b) we disregarded the questionable results in these findings. The four-column table has then the following values:

		fluorescent-serological results +	-	
hematological result according to the leukosis key	+	68	10	78
	-	95	104	199
		163	114	277

Result of the X^2 test:

X^2emp = 35.98

X^2tab = 10.82 at 0.1% I.W. for 1 FG.

Correlation coefficient: r = 0.36

The correlation is highly significant.

e). Comparison of antigen and antibody identification for the evaluation of the number of lymphocytes according to leukosis key.

In order to picture the numbers in these previous tables, we plotted in Fig. 15 the antigen and antibody curves for those numbers of lymphocytes for which the leukosis keys were considered to be negative, questionable and positive.

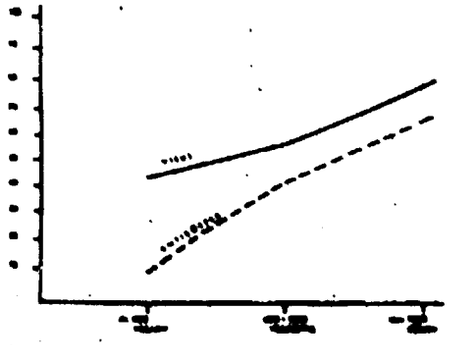

Fig. 15. Plot of the percentages of antigen (virus) or antibody identification at the critical numbers of lymphocytes in the hematological considerations.

This figure gives a compact survey of the results of the investigations of all animals older than 3 years. Of 79 investigated animals with up to 6,000 lymphocytes per mm^3 (see Table 3), which were negative according to the leukosis key, 8.9% had leukosis specific antibodies. Of the 166 animals investigated for leukosis antigen, which had also less than 6,000 lymphocytes per mm^3 (see Table 5), this antigen was identified under the fluorescence microscope in 42.8% of the cases.

In the hematologically-suspect region from 6,000 to 13,500 lymphocytes per mm^3, 40.0% of 30 animals had leukosis antibodies and 55.4% of 74 animals had leukosis antigen.

In the hematologically positive region of more than 13,500 lymphocytes per mm^3, only 66.7% of 9 animals tested had antibodies

and 77.8% of 72 animals tested had leukosis antigen.

Further we can see from the figure that in leukosis the formation of antibodies starts apparently relatively late.

IV. DISCUSSION

If further research would confirm the leukosis specificity of the described particles, which are apparently virus, then the technique with the dye-marked antibodies would be a simple method for the solution of pathogenic problems and for the diagnosis in the fight against epidemics. This method gives considerably more positive reactions than the hematological control, and might exclude the hematologically-positive cases which are not caused by leukosis. We must wait and see whether the fluorescent-serologically positive but hematologically negative animals will show in the future the characteristic lymphocytic hyperplasia.

An objection against the method, which we can at the moment not yet fully exclude, is the possibility that in the leukosis serum of cow F.-Nr.82331 there are, besides leukosis antibodies, also antibodies against other types of virus in the blood. Although the probability is low, this possibility has to be carefully investigated. We can reject this objection only when it is found possible to make experimentally highly pure test sera with purified virus.

V. SUMMARY

1. Principles and methods for marking antibodies with fluorescent dyes are described.

2. By baby mouse transfer to adapt the bovine leukosis virus to mice. The virus is therefore under experimental control.

3. By means of the "indirect method" leukosis-specific particles were found in smear samples of mouse tumors and in blood samples of cattle with tumorous or hematological leukosis, and also in blood samples of cattle which did not yet show any hematological changes. The same method in a similar application identified leukosis-specific antibodies in the serum of cattle.

4. The statistical evaluation of the results of the investigation of altogether 510 cows from 29 leukotic herds gave a highly significant Wilcoxon test for the distribution of the fluorescent-serological antigen- and antibody identification with regard to the absolute number of leucocytes in all cows over three years old. In the same way, the correlation between the fluorescent-serological result and the hematological judgment according to the leukosis key was found to be highly significant according to the χ^2-test.

- END -